The growth plate sparing effects of the selective glucocorticoid receptor modulator, AL-438

The Growth Plate Sparing Effects of the Selective Glucocorticoid Receptor Modulator, AL-438

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Abstract

Glucocorticoids (GC) are commonly used for immunomodulation and steroid replacement. Long-term use can, however, result in side effects including growth retardation in children due to their actions on growth plate chondrocytes. Recently, a non-steroidal anti-inflammatory agent (AL-438) that acts through the glucocorticoid receptor (GR) has been described. Studies with AL-438 have shown it retains full anti-inflammatory efficacy but has reduced negative effects on osteoblasts compared to those elicited by prednisolone (Pred) or dexamethasone (Dex). We have used the murine chondrogenic ATDC5 cell line to compare the effects of AL-438 with those of Dex and Pred on chondrocyte dynamics. During chondrogenesis, Dex and Pred (10^{-6}M) exposure resulted in a reduction in cell proliferation (30.3% and 18.8% respectively ($p<0.05$)) and proteoglycan synthesis (56% and 53.9% respectively ($p<0.05$)), whereas exposure to AL-438 (10^{-6}M) had no effect. LPS-induced IL-6 production in ATDC5 cells was significantly reduced by Dex or AL-438 (10^{-6}M) (58.1% and 55.4% respectively; $p<0.05$) showing that AL-438 has similar anti-inflammatory efficacy to Dex in these cells. Fetal mouse metatarsals grown in the presence of Dex were significantly shorter than control bones (18.5% shorter at day 10 ($p<0.05$) and 22.2% shorter at day 12 ($p<0.01$)) whereas AL-438 treated metatarsals paralleled control bone growth. These results indicate that the adverse effects Dex or Pred have on chondrocyte proliferation and bone growth were attenuated following AL-438 exposure, suggesting that AL-438 has a reduced side effect profile on chondrocytes compared to other GCs. This could prove important in the search for new anti-inflammatory treatments for children.

Introduction

Glucocorticoids (GC) are commonly used as anti-inflammatory or immunosuppressive treatments, and it has been estimated that 5-10% of children may require some form of GC therapy at some time in childhood [1]. Since the introduction of GCs in the treatment of rheumatoid arthritis in 1949, the therapeutic applications of these drugs have greatly broadened to encompass a large number of non-endocrine and endocrine diseases [2]. Despite intense efforts to maximise the beneficial effects and to minimise the side effects of GCs, adverse effects of GC medication are still common. Impairment of childhood growth with long-term GC was described nearly 50 years ago, however recent studies have shown that growth retardation can also occur with short-term GC therapy. In addition, it has been shown that the severity of side-effects may vary depending on the type of GC used [3,4].

Normal cartilage formation and bone growth is dependent upon chondrocyte progression through the growth plate as demonstrated by a number of null mutations that have been introduced in mice. Growth plate chondrocytes regulate the rate of longitudinal bone growth through the precise regulation of chondrocyte proliferation, hypertrophy and apoptosis [5, 6]. The mechanisms by which GC induce failure in the growth process are presently unclear but GCs act locally to inhibit longitudinal bone growth, suggesting a mechanism intrinsic to the growth plate. Baron and colleagues [7] reported that local infusion of GC into the growth plate decreased the ipsilateral tibial growth rate with no effects on the contralateral limb. In addition, both Silvestrini *et al* [8] and Smink *et al* [9] reported that GC reduced growth cartilage width, probably by inhibiting chondrocyte proliferation and increasing the apoptosis of terminal hypertrophic chondrocytes. Studies by ourselves [4,10] and others are in

agreement; reporting that GC impair chondrocyte proliferation and increase apoptosis [11, 12, 13, 14, 15]. The cellular machinery by which GC exerts control over chondrocyte dynamics is presently unclear but has been postulated to involve disruption to the growth hormone (GH) - insulin-like growth factor (IGF) axis. GH & IGF-I play essential roles in growth and development [16] with IGFs being mainly bound to IGF binding proteins (IGFBP) 1, 2 & 3 that reduce IGF bioavailability. There is some conflicting evidence that GC effects on growth may be mediated via changes in GH sensitivity as well as changes in IGFBP expression [17] and IGF-I dependent phosphorylation events [18, 19, 20]

Recently, a non-steroidal inflammatory agent (AL-438) that acts through the glucocorticoid receptor (GR) has been described [21]. Studies with AL-438 using the carrageenan-induced paw edema assay and the adjuvant arthritis models in rats have shown it retains full anti-inflammatory efficacy but has reduced negative effects on bone compared to those elicited by Prednisolone (Pred) [21]. It is presently unknown whether AL-438 has less detrimental effects on growth plate chondrocytes than other commonly used anti-inflammatory steroids. In this context, the effects of AL-438 have been compared with those of Dexamethasone (Dex) and Pred using the chondrogenic ATDC5 cell line and the fetal mouse metatarsal organ culture model. The ATDC5 cell line established by Atsumi et al. [22] from the mouse teratocarcinoma cells AT805, mimics many of the events described for differentiation of epiphyseal chondrocytes This line has less phenotypic diversity than cell cultures of primary chondrocytes, and also allows the study of the differentiation of mesenchymal cells into chondrocytes and the terminal differentiation of proliferating to hypertrophic chondrocytes [4]. The fetal mouse metatarsal explant culture provides

a more physiological model for studying bone growth. It maintains cell-cell and cell-matrix interactions and the direct assessment of bone growth and histological architecture can be determined [23, 24].

Materials and Methods

Chondrocyte cell culture

The ATDC5 chondrocyte cell line was obtained from the RIKEN cell bank (Ibaraki, Japan), and cells were cultured at a density of 6000 cells per cm^2 in differentiation medium in multi-well plates [25] (Costar, High Wycombe, UK). Differentiation medium contained DMEM/Ham's F12 (Invitrogen, Paisley, UK), 5% FCS (Invitrogen), $3x10^{-8}$M sodium selenite and 10μg/ml human transferrin (Sigma, Poole, UK) and insulin (10μg/ml; Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2/95% air for up to 17 days and the medium was changed every second day after reaching confluency (day 6).

Gene expression

In order to study and compare the effects of AL-438 (Ligand Pharmaceuticals, San Diego, USA), Dex and Pred (Sigma) during both chondrogenesis (days 6-10) and terminal differentiation phases (days 13-17), all three compounds were added to the cells at the beginning of both time points (days 6 and 13, respectively) at a final concentration of 10^{-6}M [4] in 0.01% dimethyl sulfoxide (DMSO). Control cultures contained 0.01% DMSO only. Cells were grown in the presence of the compounds for 4 days, at which point RNA was extracted, reverse transcribed and analysed for aggrecan gene expression by semiquantitative (sq) and quantitative (q) RT-PCR.

RNA extraction

Total RNA was extracted from chondrocytes by repeated aspiration though a 25-gauge syringe needle in 1ml Ultraspec (Biotecx, Houston, Texas, USA). After extraction with chloroform, RNA in the aqueous phase was purified with isopropanol and bound to 50µl RNA Tack resin, before washing with 75% ethanol. The RNA was then eluted in 100µl nuclease free water and treated with DNase. For each sample the 260/280 ratio was determined, and the samples diluted to a concentration of 60ng/µl and stored at -80^{o}C.

Semiquantative and Quantitative RT-PCR

Gene expression was analysed by both sq [26] and qPCR. RNA samples or blanks (containing nuclease-free water in place of RNA) were reverse transcribed in 20µl reactions with 200ng random hexamers and 200U Superscript II reverse transcriptase using the Superscript preamplification protocol (Invitrogen). sqPCR was performed in 20µl reactions containing cDNA equivalent to 10ng RNA and 200nM gene-specific primers (Aggrecan: forward 5' CGAGAATGACACCTGCTAGG 3', reverse 3' AAGAAGACAGGACCAGGAAGG 5'; 18S: Unknown, purchased from Ambion) in 10XPCR buffer [26]. The cycling profile was 1 min at 92^{o}C (first cycle 2 min), 1 min at 55^{o}C and 1 min at 72^{o}C. The number of cycles varied between genes to ensure that the reactions were in the exponential phase. Reaction products were analysed on 1.5% agarose gels in the presence of ethidium bromide (250µg/l) and a digital image of each gel was captured using a gel documentation system. qPCR was performed using the Stratagene Mx3000P real-time QPCR system (Stratagene, California, USA). Primers for Aggrecan were obtained from SuperArray Bioscience (Maryland,

USA; sequence unknown) and primers for GAPDH were designed using the software programme Primer3 (Whitehead Institute for Biomedical Research) (forward 5' TGAGGCCGGTGCTGAGTATGTCG 3', reverse 5' CCACAGTCTTCTGGGTGGCAGTG 3'). cDNA (5ng) was amplified in triplicate using the Platinum SYBR Green qPCR SuperMix (Invitrogen, Paisley, UK) under the following conditions: cDNA was denatured for 2 minutes at 95 ^{o}C, followed by 40 cycles consisting of 15 s at 95^{o}C and 30 s at 60^{o}C and 1 cycle consisting of 1 min at 95^{o}C and 30 s at 60 ^{o}C. Each reaction contained 10µl cDNA, 25µl Platinum SYBR Green qPCR Super-Mix, 1µl ROX Reference Dye, and 250nM primer in a total volume of 40µl. Fold changes, normalised for the expression of GAPDH, were calculated using the comparative method [27].

Rate of chondrocyte proliferation

Compounds (10^{-6}M, 10^{-7}M or 10^{-8}M) were added to the cells during both time points as previously described, and were incubated for 24h. Chondrocyte proliferation was assessed by incubating the chondrocytes with 0.2µCi/ml [^{3}H]thymidine (37MBq/ml; Amersham Pharmacia Biotech, Bucks, UK) for the last 2h of the incubation period and the amount of radioactivity incorporated into trichloroacetic acid insoluble precipitates was measured. The effect of AL-438 on chondrocyte number was also determined by incubating the cells with AL-438, Pred, or Dex for 48h and counting the cells directly in a haemocytometer chamber.

Alkaline Phosphatase activity and Proteoglycan production

Dex, Pred and AL-438 (10^{-6}M) or 0.01% DMSO were added to ATDC5 cells at day 6 or day 13 for 96h. For analysis of alkaline phosphatase (ALP) activity, cells were rinsed with phosphate buffered saline (PBS) and lysed with 0.9% NaCl and 0.2%

Triton X-100 and centrifuged at 12000*g* for 15min at 4^{o}C. The supernatant was assayed for protein content and ALP activity as a measure of cell number and chondrocyte differentiation respectively. The protein content of the supernatant was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories; Hemel Hempstead; UK) and gamma globulin was used as a standard. For analysis of proteoglycan production, ATDC5 cells were washed twice with PBS, fixed in 95% methanol for 20min and stained with 1% Alcian Blue 8GX in 0.1M HCl overnight and rinsed with distilled water. Alcian Blue stained cultures were extracted with 1ml 6M Guanidine-HCl for 6h at room temperature and the optical density (O.D) was measured at 630nm using a Jenway 6105 spectrophotometer [28].

Apoptosis

Apoptosis was measured using the Caspase-3 Apoptosis assay, which was used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Dex, Pred and AL-438 (10^{-6}M) were added to the cells during chondrogenesis or terminal differentiation periods for 24h. As a positive control, cells were incubated as above with 10ng/ml TNFα (Autogen Bioclear, Wiltshire, UK).

Determination of anti-inflammatory efficacy of AL-438 in ATDC5 cells

ATDC5 cells were grown in 10µg/ml lipopolysaccharide (LPS) (Lot 104K4036; from EColi 0127:B8; Sigma) with or without Dex or AL-438 (10^{-6}M) for 24h. The medium was removed and analysed for levels of IL-1, IL-6 and TNF-α by Luminex technology (assay completed by Dr Alistair Gracie, Division of Immunology, Infection and Inflammation, University of Glasgow).

Fetal metatarsal organ culture

The middle three metatarsals were aseptically dissected from 18-day-old embryonic Swiss mice that had been killed by decapitation. The experimental protocol was approved by Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals. Bones were individually cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2 in 24-well plates (Costar) for up to 12 days. Each well contained 300µl of α-MEM without nucleosides (Invitrogen) supplemented with 0.2% BSA Cohn fraction V (Sigma), 0.1mmo/l β-glycerophosphate (Sigma), 0.05mg/ml L-absorbic acid phosphate (Wako, Japan), 0.292mg/ml L-glutamine (Invitrogen), 0.05mg/ml gentamicin (Invitrogen) and 1.25µg/ml fungizone (Invitrogen). Dex or AL-438 were both added at a final concentration of 10^{-6}M in 0.01% DMSO to the bones and the medium was changed every second or third day. The control groups (0.01% DMSO only) and experimental groups contained 6 metatarsals each.

Morphometric analysis

Images were taken of the metatarsals every second or third day of culture using a digital camera attached to a Nikon TE300 microscope (Figure 1). The total length of the bone was determined using Image Tool (Image Tool version 3.00, University of Texas Health Life Science Centre, San Antonio, TX). All results are expressed as a percentage change from harvesting length, which was regarded as baseline to demonstrate the rate of growth over time. For the determination of the size (in the direction of longitudinal growth) within the growth region of the distinct chondrocyte maturational zones, the 12-day-old metatarsals were fixed in 70% ethanol,

dehydrated, and embedded in paraffin wax [29]. Wax sections (10μm in thickness) were reacted for ALP activity for the demarcation of the hypertrophic and proliferating zones. Sections were also stained with von Kossa and hematoxylin and eosin using standard protocols to identify the zone of cartilage mineralization [10]. Images of the stained metatarsals were captured and the size of the ALP-negative proliferating zone was determined (proliferating zone = total length – (hypertrophic zone + mineralizing zone). The size of the hypertrophic zone was determined by subtracting the von Kossa stained mineralizing zone from the ALP-positive zone, and the size of the mineralizing zone was determined directly from the von-Kossa stained sections.

Cell proliferation: BrdU and PCNA staining

BrdU (Sigma) was added (1mg/ml final concentration) to the culture medium of the metatarsals for the last 6h of culture on day 12. At the end of the incubation period, the tissue was washed in PBS and fixed in 70% ethanol, dehydrated, and embedded in paraffin wax. Sections 10μm in thickness were cut along the longitudinal axis, and chondrocyte nuclei with incorporated BrdU were detected using a cell proliferation kit (Amersham Biosciences, UK) Briefly, sections were incubated with a monoclonal antibody to BrdU diluted 1:50 in PBS for 1h. After washing, the sections were incubated for an additional 1h in peroxidase anti-mouse IgG2a and then reacted with diaminobenzidine before being dehydrated and mounted in DPX. The total number of BrdU positive chondrocytes within the proximal and distal growth regions was determined, and normalized to section area. Sections were also stained with an antibody to proliferating cell nuclear antigen (PCNA) (Sigma) using a standard indirect immunoperoxide protocol as described for BrdU (above).

Statistical Analysis

Data were analysed by one-way analysis of variance. All data are expressed as the mean ± S.E.M of at least 6 replicates within each experiment, and statistical analysis was performed using Minitab (Minitab 14; PA, USA). $P<0.05$ was considered to be significant.

Results

Effect of AL-438 on ATDC5 cell number and proliferation

During chondrogenesis exposure to Dex or Pred for 24h caused a concentration-dependent reduction in [^{3}H]-thymidine incorporation (54.5% and 29.2%, respectively, at 10^{-6}M ($p<0.05$)), whereas exposure to AL-438 had no significant effect at any concentration tested (Figure 1a). Consequently, 10^{-6}M Dex, Pred or AL-438 was used for all future experiments. Counting the cells directly in a haemocytometer chamber supported these results, with Dex and Pred causing a significant reduction in cell number at 10^{-6}M (36.2% and 36.8% respectively ($p<0.05$)), and AL-438 having no significant effect (Figure 1b). During the terminal differentiation phase (days 13-17), none of the compounds significantly altered chondrocyte proliferation or cell number (data not shown).

Effect of AL-438 on proteoglycan production, ALP activity and expression of chondrocyte marker genes in ATDC5 cells

In comparison with control cultures during the chondrogenesis period, exposure to Dex and Pred for 96h caused a significant reduction in proteoglycan synthesis (56% and 54%, respectively; $p<0.05$), whereas exposure to AL-438 had no effect (Figure

1c). No significant differences between treatments were noted during terminal differentiation (data not shown). The effect of AL-438 on terminal chondrocyte differentiation was assessed by ALP activity. During chondrogenesis, ALP activity was significantly increased after exposure to Dex, Pred and AL-438 ($p<0.05$) (Figure 1d), but no significant differences were observed during terminal differentiation (data not shown). Semiquantitative PCR revealed that gene expression levels of aggrecan were reduced in Dex treated cells, but were unchanged in Pred or AL-438 treated cells (Figure 2a). qPCR analysis confirmed a significant reduction in aggrecan expression with Dex (3.5-fold reduction; $p<0.05$), and also revealed a significant reduction in aggrecan with Pred (1.36-fold; $p<0.05$) (Figure 2b). Caspase-3 activity was unchanged in Dex, Pred and AL-438 treated cells compared to controls during both chondrogenesis and terminal differentiation (data not shown). TNF-α did cause a significant increase in Caspase-3 activity as expected.

Anti-inflammatory efficacy of AL-438 in ATDC5 cells

LPS induced IL-6 production in ATDC5 cells and this induction was reduced by the co-incubation Dex or AL-438 by 58.1% and 55.4%, respectively. These results were significantly different ($p<0.05$) from IL-6 levels in LPS-only treated cells, but not significantly different from each other ($p>0.05$) (Figure 3). Interestingly, levels of IL-1 and TNF-α were undetected in control and test samples. These results demonstrate that AL-438 has a similar anti-inflammatory efficacy to Dex in ATDC5 cells.

Longitudinal bone growth and assessment of chondrocyte maturational zone sites

Until day 7 of culture, fetal mouse metatarsals treated with Dex or AL-438 grew at a similar rate to controls. However, subsequently, Dex treated metatarsals were

significantly shorter than control bones (18.5% shorter at day 10 ($p<0.05$) and 22.2% shorter at day 12 ($p<0.01$)), whereas AL-438 treated metatarsals continued to parallel control bone growth (Figure 4). The lengths of the proliferating, hypertrophic and mineralizing zones were measured on histological sections of 12-day-old metatarsals (Table 2; Figure 5). Although, the lengths of the proliferating and hypertrophic zones did not significantly change between treatments, Dex significantly reduced the length of the mineralizing zone (57%; $p<0.05$).

Cell proliferation in metatarsals: BrdU and PCNA staining

The number of proliferating chondrocytes within the metatarsals was significantly reduced by Dex (78.9%; $p<0.05$) and AL-438 (83.9%; $p<0.05$) (Figure 6). Interestingly, the majority of positive staining was observed within the cells of the perichondrium, with only a small number of proliferating chondrocytes being detected within the proliferative zone.

Discussion

Glucocorticoids are the most effective anti-inflammatory agents known. However, the long-term use of these steroids leads to a large number of side effects, including growth retardation in children. The exact mechanisms by which GC induce failure in the growth process are presently unclear, but early studies involving local infusion of GC directly into the growth plate decreased the ipsilateral tibial growth rate with no effects on the contralateral limb, suggesting a mechanism intrinsic to the epiphyseal growth plate [7]. It has also been shown that GC reduce growth plate cartilage width, and it has been suggested that this is due to GC inhibiting chondrocyte proliferation and increasing the apoptosis of terminal hypertrophic chondrocytes [8, 9].

The search for a novel GC that has the anti-inflammatory properties of conventional steroids without one or more of the side-effects has been a long-standing goal of the field. Much effort has been spent on modifying the steroid backbone to achieve this, however, these efforts have been met with little success. Deflazacort, a D-ring substituted steroid, was originally hailed as a powerful anti-inflammatory molecule exhibiting reduced activity in bone and glucose metabolism [29]. Initially, clinical data supported this notion [30, 31]. However, subsequent trials that adjusted the steroid dose to maintain equivalent anti-inflammatory efficacy usually needed higher levels of deflazacort. Unfortunately, at these higher doses, the advantages of deflazacort disappeared. The field was revived by the discovery that in order to repress pro-inflammatory genes, the GR binds directly to specific transcription factors (NFκB and AP-1) which can up- or down-regulate inflammatory genes [32, 33]. This represented a unique mechanism that was genetically separable from transcriptional activation, and initiated the search for ligands that could induce transcriptional repression, but reduce transcriptional activation.

AL-438 is a specific, non-steroidal ligand for the GR that exhibits a unique profile both *in vivo* and *in vitro*. It retains full anti-inflammatory activity but has reduced negative effects on osteoblasts compared to Pred [21, 34, 35]. Interestingly, incubating ATDC5 cells with AL-438 had no effect on cell proliferation, cell number or proteoglycan synthesis, whereas, as found in previous studies, both Dex and Pred significantly reduced ATDC5 proliferation, cell number and proteoglycan synthesis [4, 36]. The lack of effect of Dex on ATDC5 apoptosis is in agreement with previous studies [4] but at variance with other chondrocyte studies [18] suggesting a cell type

dependent effect. Studies measuring the bone turnover marker osteocalcin in the human osteoblast cell line MG-63 have shown that AL-438 is unable to inhibit osteocalcin expression as efficiently as Pred [24]. In addition, AL-438 has been shown to exhibit a weak inhibition of osteoprotegerin in MG-63 cells compared to other GCs [21]. The differential effect on ATDC5 cell progression between AL-438 and Dex and Pred may be due to the fact that AL-438 is qualitatively different in terms of its ability to activate or repress gene expression. Interestingly, none of the compounds had an effect on ATDC5 maturation in terms of proliferation, ALP activity or proteoglycan production during the terminal differentiation period, a finding that has been reported previously [4].

The mechanism of action of AL-438 is based on the hypothesis that many genes involved in undesirable side effects are upregulated (such as enzymes in lipid and muscle metabolism), whereas many pro-inflammatory genes (i.e. IL-1, IL-6 and TNF-α) are repressed. It has been hypothesised that upon binding to the GR, AL-438 induces structural changes in the receptor that are different than those induced by other GR ligands such as Pred or Dex [21]. These structural changes promote reduced co-factor interactions between the GR and co-factors such as PGC-1, which is involved in hepatic glucose production, but do not change the interactions between GRIP-1 and the GR, a co-factor which plays a role in the repression of pro-inflammatory genes [21]. This hypothesis may explain the reported maintenance of anti-inflammatory activity with AL-438 [21] but also its reduced effects on ATDC5 cells as found in this study. The proposed mechanism of action of AL-438 is also supported by the finding that as opposed to Dex, AL-438 does not have a detrimental effect on metatarsal bone growth. This finding confirms bone-sparing results from

previous studies on rats treated with Pred or AL-438, which used calcein and tetracycline labelling to show that whilst Pred significantly reduced the rate of cortical bone formation, AL-438 had no effect [21]. The fact that the length of the mineralizing zone, but not the proliferating or hypertrophic zones, was significantly shorter in Dex treated bones suggests that Dex is acting directly on the terminally differentiated chondrocytes to reduce overall bone length. A similar finding has previously been found by our group [10] and others [37], and may be caused by Dex interfering with genes involved in the mineralization process. The finding that Dex or AL-438-treated metatarsals had a similar number of proliferating chondrocytes was surprising when compared to the *in vitro* proliferation data, and could be explained by the suggestion that the activity of transcriptional activators such as AL-438 can vary under different conditions and in different tissue types [38]. This data intriguingly suggests that bone growth within the metatarsal model is not wholly dependent on chondrocyte proliferation as growth retardation was not observed in the AL-438 treated metatarsals despite lowered proliferation. Other aspects of chondrocyte dynamics such as alterations in hypertrophy may be involved and this requires further investigation.

The finding that AL-438 maintains a similar anti-inflammatory efficacy to Dex in ATDC5 cells was important, as it proved that AL-438 was still fully efficacious as an anti-inflammatory agent in chondrocytes despite the fact that it was much less harmful than Dex or Pred. The anti-inflammatory efficacy of AL-438 has previously been demonstrated both *in vivo* using the rat carrageenan-induced paw edema assay and in *vitro* in HepG2 cells and human skin fibroblasts [21].

To compensate for the fact that AL-438 is slightly less potent than Pred a saturating dose of 10^{-6}M was used for all compounds in this study. In addition, this pharmacological concentration of Dex and Pred is similar to that found in chronic GC therapy and has been shown previously by us, and others, to inhibit both chondrocyte proliferation and longitudinal bone growth [4, 10]. At 10^{-6}M the compounds fully occupied the receptor, therefore allowing their full efficacy as anti-inflammatory agents to be measured. Although AL-438 has been shown to have similar affinity for the GC-receptor across a wide array of tested cell types [21, plus unpublished data] it also exhibits some affinity for the mineralocorticoid receptor at this concentration. This affinity is comparable to that of 10^{-6}M Pred, and when tested against a variety of other nuclear and non-nuclear receptors, kinases and enzymes, AL-438 displayed no affinity up to a concentration of 10μM (unpublished). In addition to interactions with its receptor, GC are also known to have non –genomic effects that are not mediated by the GC-receptor [39]. Although all indications are that AL-438 acts exclusively through the GC-receptor [21] further studies are required to establish the precise cellular events involved in AL-438 actions. Nevertheless the data from this study suggests that AL-438 may separate anti-inflammatory activity from a number of bone growth-related side effects, including chondrocyte proliferation and longitudinal bone growth. endorses the principle for searching for novel anti-inflammatory agents with minimal side effects. The reduced skeletal side effect profile of this novel selective glucocorticoid receptor modulator compared to other GCs could prove important in the search for new anti-inflammatory treatments for children.

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Table 1 – Lengths of the proliferating, mineralizing and hypertrophic zones.

Treatment	Mineralizing zone (mm)	Hypertrophic zone (mm)	Proliferating zone (mm)
Control	1.29 +/- 0.08	0.68 +/- 0.09	0.84 +/- 0.02
Dexamethasone	0.74 +/- 0.19 *	0.77 +/- 0.12	0.71 +/- 0.05 [a]
AL-438	1.30 +/- 0.07	0.56 +/- 0.06	0.80 +/- 0.03

Data are expressed in mm ± sem (n = 6). *P<0.05 compared with controls; aP = 0.06 compared with controls.

Figure 1 (A) Effect of Dex, Pred and AL-438 on cell proliferation as assessed by [^{3}H]-thymidine uptake during chondrogenesis. Effect of Dex (♦ and short dashed lines); effect of Pred (□ and long dashed lines); effect of AL-438 (▲ and solid line). Data are expressed as means ± sem (n = 6). ***P<0.001; *P<0.05 compared with control treated cells. (B) Effect of Dex, Pred and AL-438 (all 10^{-6}M) on cell number during chondrogenesis. Data are expressed as means (10^4) ± sem (n = 6). (C) Effect of Dex, Pred and AL-438 (all 10^{-6}M) on proteoglycan production as assessed by Alcian Blue staining in ATDC5 cells during chondrogenesis. *P<0.05 compared with control and AL-438 treated cells. (D) Effect of Dex, Pred and AL-438 (all 10^{-6}M) on ALP activity in ATDC5 cells during chondrogenesis. All data are expressed as means ± sem (n = 6). *P<0.05 compared with control treated cells.

Figure 2 (A) Semiquantitative RT-PCR analysis of the expression of Aggrecan in ATDC5 cells treated with Dex, Pred or AL-438 (all 10^{-6}M). Dex, but not Pred or AL-438 reduces the expression of Aggrecan. (B) Aggrecan mRNA levels as measured by quantitative PCR in ATDC5 cells. All data are shown as the mean relative gene expression ± sem (n=3) and normalised to GAPDH mRNA levels. *P<0.05 compared with control treated cells.

Figure 3 - LPS-induced IL-6 production in ATDC5 cells treated with Dex or AL-438 (all 10^{-6}M). Data are expressed as means ± sem (n = 6). *P<0.05 compared with control and LPS treated cells.

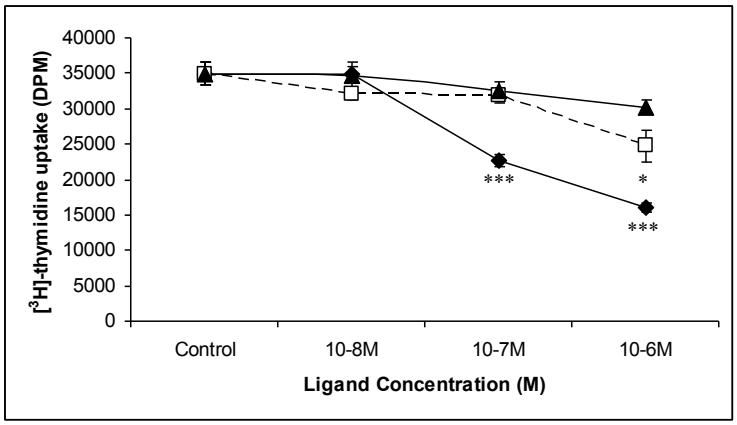
Figure 4 - Effect of Dex or AL-438 (10^{-6}M) on the growth of metatarsals from 18-day old fetal Swiss mice over a 12-day period. Data are expressed as mean ± sem (n = 6). **P<0.01 compared with controls; *P<0.05 compared with controls.

Figure 5 – Digital image of d10 fetal mouse metatarsal section reacted for ALP activity, showing staining within both the mineralising and hypertrophic zone. The location of the proliferating (PZ), mineralizing (MZ), and hypertrophic (HZ) zones are also illustrated.

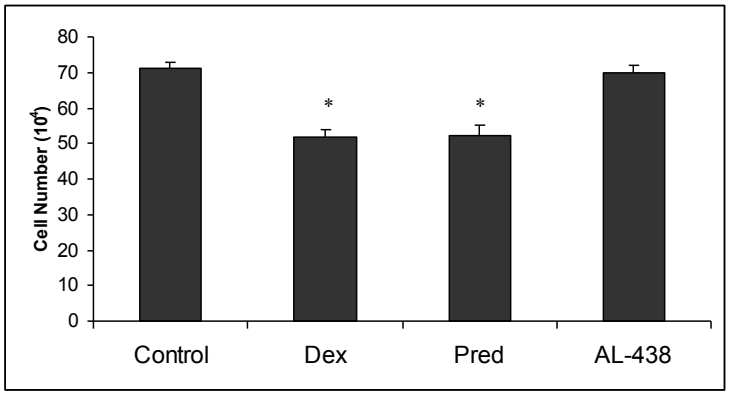
Figure 6 – Effect of Dex and AL-438 on the number of proliferating chondrocytes present in metatarsals (as represented by BrdU positive cells/section area). Data are expressed as mean ± sem (n = 6). ***P<0.001 compared with controls.

Figure 1

A



B



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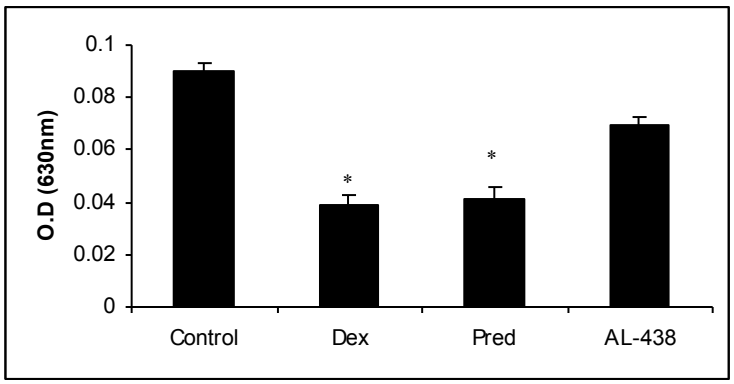


D

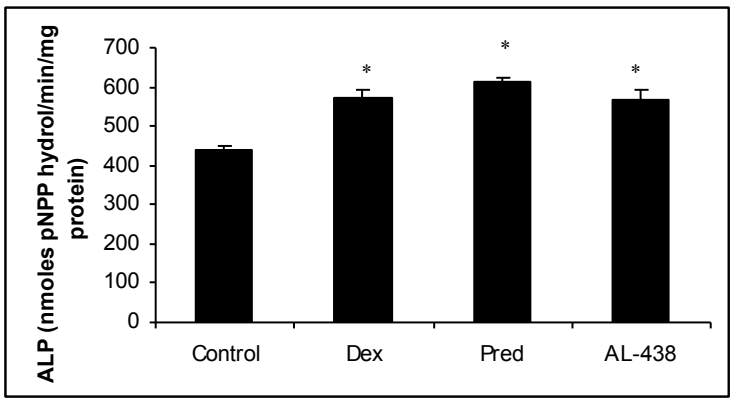
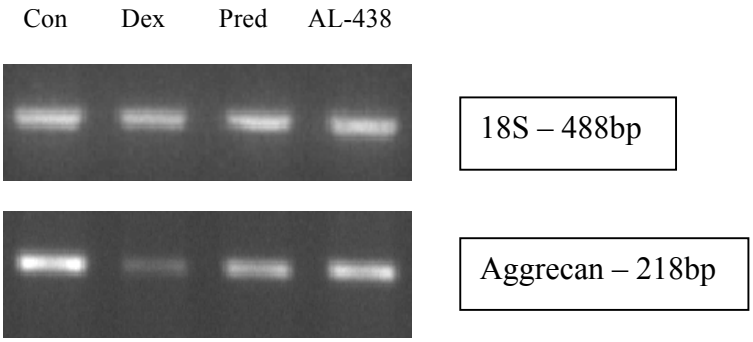


Figure 2

A


Con
Dex
Pred
AL-438
18S – 488bp
Aggrecan – 218bp

B

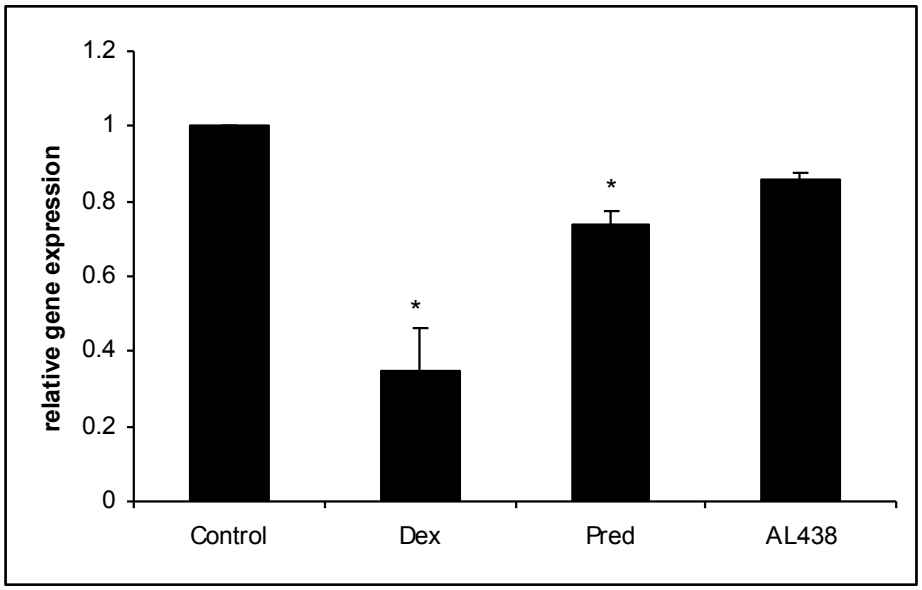

relative gene expression
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
Dex
Pred
AL438

Figure 3

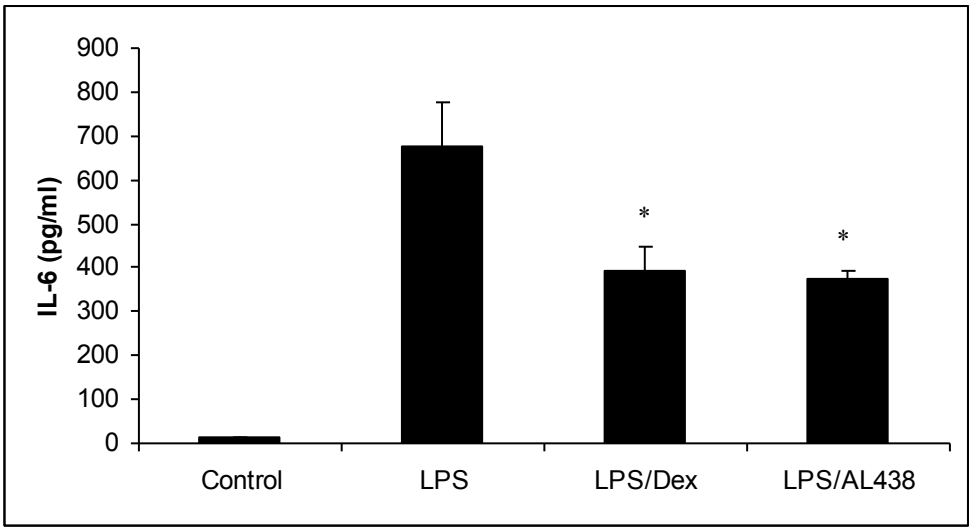


Figure 4

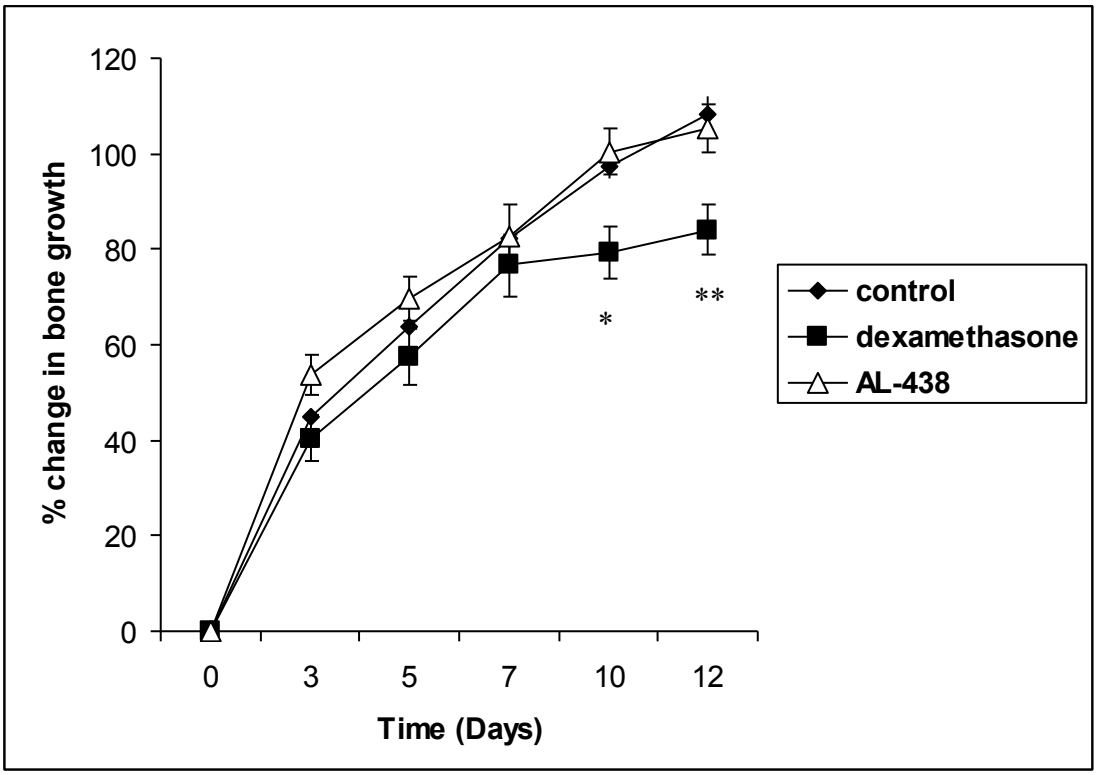


Figure 5

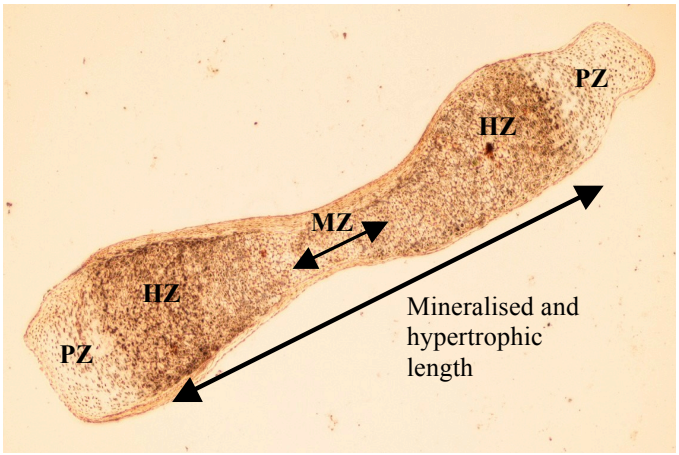


Figure 6